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Requirement of the Initial Production of Gamma Interferon in the Generation of Protective Immunity of Mice against *Listeria monocytogenes*

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Protective immunity of mice against *Listeria monocytogenes*, which is mediated mainly by gamma interferon (IFN-γ)-producing T cells, was induced by immunization with viable bacteria but not with killed bacteria. By comparing mice immunized with either viable or killed *L. monocytogenes*, it was found that IFN-γ was produced at the initial stage only after immunization with viable bacteria. This finding prompted us to investigate the effect of neutralizing the IFN-γ on the final generation of protective T cells against *L. monocytogenes*. When endogenous IFN-γ was neutralized by administration of anti-IFN-γ monoclonal antibody for the initial 2 days in mice immunized with viable bacteria, the generation of protective T cells on day 6 was completely blocked, as revealed by T-cell adoptive transfer. The generation of listeria-specific IFN-γ-producing T cells was also abolished. These results clearly demonstrated that endogenous IFN-γ, which is produced at the initial stage of immunization, actually plays a critical role in the generation of protective T cells against *L. monocytogenes* in vivo. Moreover, this study suggested that the lack of IFN-γ-inducing ability is responsible for the inability of killed *L. monocytogenes* to induce protective T cells in mice.

Listeria monocytogenes, a gram-positive facultative intracellular bacterium, has been used widely to study cell-mediated immunity against bacterial infection. In mice immunized with viable *L. monocytogenes*, $CD4^+$ T cells contribute to the acquired protective immunity by producing cytokines, especially macrophage-activating gamma interferon (IFN-γ) (18, 38). Among heterogeneous sets of $CD4^+$ T cells, the protective T cells are regarded as Th1 type, showing a unique profile of cytokine production different from that of the Th2 type of cells (22, 23). Th1 cells are characterized particularly by the ability to produce IFN-γ (15, 22). It has been established that IFN-γ is critically important in the expression phase of protection against *L. monocytogenes*. By using an anti-IFN-γ monoclonal antibody (MAb), Buchmeier and Schreiber indicated that endogenous IFN-γ production was required for the resolution of *L. monocytogenes* infection (2). Nakane et al. also reported that neutralization of endogenous IFN-γ resulted in the impairment of not only primary defense but also the secondary defense against challenge infection (24). The direct action of IFN-γ on macrophage activation for an enhanced killing of *L. monocytogenes* has been well elucidated in vitro (36). All these lines of evidence clearly indicate the importance of IFN-γ in the expression phase of both nonspecific and antigen-specific protection against this intracellular bacterium.

By using a number of nonliving mycobacterial preparations, Orme has reported that T-cell-dependent protective immunity could not be induced by killed bacteria or bacterial cell walls though they were highly immunogenic for the generation of delayed-type hypersensitivity (DTH) effector T cells (28, 29). We have also obtained similar results with *L. monocytogenes* (11, 21, 38) and reported that the protective T cells were characterized by the ability to produce a large amount of IFN-γ in an antigen-specific manner while T cells mediating only DTH were not (13, 38). The dissociated development of DTH-mediating T cells and those mediating both DTH and protective immunity appears to have been generally accepted (19). One explanation for this dissociation may be the difference in the antigen(s) recognized by T cells between viable and nonliving bacteria. In our own study, however, an antigen(s) existing in both viable and killed bacteria was recognized by IFN-γ-producing T cells obtained from mice immunized with viable bacteria (14). An alternative explanation is that the nonliving preparation is incapable of inducing some factor(s) which plays a pivotal role for the development of protective T cells in the immunized host.

We have observed that IFN-γ mRNA expression could be induced after immunization with virulent strains of *L. monocytogenes*, while nonvirulent strains which are incapable of inducing protective immunity did not induce mRNA expression of IFN-γ in vivo (39). A similar difference in IFN-γ induction was observed between viable and killed *Mycobacterium bovis* BCG in vitro. Only viable BCG induced the natural killer (NK)-cell-dependent IFN-γ production at the early stage of immunization (40). Based on these findings, we have postulated that the endogenously produced IFN-γ at the initial phase may be one of the critical factors required for the generation of protective T cells. In order to confirm this assumption, we first compared the IFN-γ induction between groups of mice immunized with viable or killed *L. monocytogenes*, and then we determined the effect of neutralizing the initial IFN-γ on the generation of protective T cells against *L. monocytogenes* in vivo.

MATERIALS AND METHODS

Mice. Female C3H/He mice raised under specific-pathogen-free conditions were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). The mice were maintained at least for 1 week and were used for experiments at 8 to 10 weeks of age.

L. monocytogenes. *L. monocytogenes* EGD, which has been maintained for a long time, was used throughout the study. The bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C for 16 h, washed repeatedly, resuspended in phosphate-buffered saline (PBS), and stored at −80°C in small aliquots. The number of viable bacteria was determined by plating the dilutions onto tryptic soy agar (Difco) and counting the colonies after overnight incubation

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in each experiment. Heat-killed bacteria were prepared by heating viable *L. monocytogenes* at 74°C for 90 min (38).

Anti-mouse IFN-γ antibody. Hybridoma R4-6A2, which secretes rat immunoglobulin G1 (IgG1) MAb against murine IFN-γ (35), was cultured in serum-free RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 2% TCH (a serum replacement; CELOX Corporation, Hopkins, Minn.). The MAb in the culture supernatant was partially purified by ammonium sulfate precipitation. The characteristics of the MAb were described previously (35).

RNA extraction. Mice were intravenously injected with 10^3 viable *L. monocytogenes* cells or 10^3 or 10^8 killed *L. monocytogenes* cells. One day after the injection, total cellular RNA in spleens was extracted by a previously described method (40). Briefly, mice were sacrificed and spleens were homogenized at room temperature in denaturing solution, which was a mixture of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), water-saturated phenol, and 2 M sodium acetate (pH 4.0) (1:1:0.2, vol/vol/vol). The homogenate was left for 5 min on ice and mixed with chloroform. After vigorous agitation, the homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C, and the aqueous phase was collected. RNA was precipitated by addition of the same volume of isopropanol and cooling at −20°C overnight. RNA was collected by centrifugation at 10,000 × *g* for 10 min at 4°C, rinsed once with 75% ethanol, dried, and finally dissolved in distilled water. The amount of RNA was quantified by determining the optical density at 260 nm with GeneQuant (Pharmacia LKB Biochem Ltd., Cambridge, United Kingdom).

Reverse transcription (RT) and RT-PCR. Two micrograms of total cellular RNA from spleens in experimental groups were used for cDNA synthesis carried out with random primers [pd(N)$_6$; Pharmacia Biotechnology AB, Uppsala, Sweden] and SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, Md.) as described previously (40). After heat inactivation of the enzyme (95°C, 3 min), an aliquot of each cDNA was subjected to amplification cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min with a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.). The PCR mixture contained 0.03 U of *Taq* DNA polymerase (Promega, Madison, Wis.) per μl, 1 mM $MgCl_2$, and 0.2 mM deoxynucleoside triphosphate (Pharmacia). Samples were subjected to 22 and 26 cycles for amplification of β-actin and IFN-γ, respectively. In the preliminary experiments, the optimal cycles for each primer set were assessed based on the linear portion of the curve for the amount of products. The sequences of sense and antisense PCR primers used for amplification are as follows, and the predicted size of PCR product is indicated in parenthesis: 5′-AGCGGCTGACTG AACTCAGATTGTAG-3′ and 5′-GTCACAGTTTTCAGCTGTATAGGG-3′ for IFN-γ (244 bp) and 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ for β-actin (349 bp). Eight microliters of each PCR product in 50 μl of reaction mixture was electrophoresed by using a 1% low-melting-temperature agarose gel (Wako Pure Chemicals, Osaka, Japan) in Tris-acetate-EDTA (TAE) buffer supplemented with ethidium bromide. The product was visualized on a UV transilluminator and photographed.

Immunization of mice. Mice were intravenously immunized with 10^3 viable *L. monocytogenes* cells or 10^3 or 10^8 killed *L. monocytogenes* cells. Six days after the immunization, mice were intravenously challenged with 10^4 viable *L. monocytogenes* cells, and the protective immunity was determined by counting the number of CFU in spleens 2 days later. In other experiments, mice were intravenously immunized with 10^3 viable *L. monocytogenes* cells and treated with 400 μg of anti-IFN-γ MAb by intraperitoneal injection three times at −2, 24, and 48 h. An equivalent amount of normal rat IgG (Sigma Chemical Company, St. Louis, Mo.) was used as a control for the nonspecific effects of injecting rat IgG.

Preparation of culture supernatants and sera. Six days after immunization with viable *L. monocytogenes*, T cells were obtained from spleen cells by passage through a column packed with nylon wool fiber (Wako). Non-nylon-wool-adherent T cells (2.5×10^6/ml) were suspended in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Gibco BRL Life Technologies, Inc., Grand Island, N.Y.), 25 μg of gentamicin/ml, 5×10^{-5} M 2-mercaptoethanol, 5 g of HEPES/liter and 2 g of $NaHCO_3$ (Wako) per liter. The cells were stimulated with 10^8 cells of heat-killed *L. monocytogenes* in the presence of normal splenic adherent cells as antigen-presenting cells (APC). The culture supernatants were collected 18 h after stimulation for quantitative analysis of IFN-γ. In some experiments, sera were collected 2 days after immunization and used for titration of IFN-γ.

IFN-γ assay. IFN-γ titer was determined by enzyme-linked immunosorbent assay (ELISA) as previously described (40). Briefly, the supernatants, sera, or authentic murine recombinant IFN-γ were added to the wells of enzyme immunoassay plates (Costar, Cambridge, Mass.) precoated with 1.5 μg of rat anti-mouse IFN-γ MAb (R4-6A2) per ml and 0.5% bovine serum albumin in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After incubation for 60 min, the plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween) and incubated with rabbit anti-mouse IFN-γ polyclonal antibody for 60 min. After being washed with PBS-Tween, peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories, Inc., San Francisco, Calif.) was added. After incubation for 60 min, the plates were washed and 100 μl of 0.4-mg/ml orthophenylenediamine in phosphate-citrate buffer (pH 5.0) containing 0.03% H_2O_2 was added as a substrate solution. The reaction was terminated by adding 50 μl of 2.5 N H_2SO_4, and A_{490} was measured. IFN-γ titers were expressed as units per milliliter.

ELISPOT assay. To enumerate the antigen-specific IFN-γ-producing cells, an ELISPOT assay was carried out as described previously (13). Briefly, non-nylon-wool-adherent T cells were stimulated with heat-killed *L. monocytogenes* in the presence of APC for 18 h. After being washed, the cells were resuspended in RPMI 1640 medium containing 10% FCS and seeded at concentrations of 5×10^3 to 5×10^5 cells/100 μl/well in nylon-based 96-well plates (Costar) which had been coated with rat anti-IFN-γ MAb and blocked with RPMI 1640 medium containing 10% FCS. After further incubation for 20 h, the plates were thoroughly washed with PBS-Tween and incubated with rabbit anti-mouse IFN-γ polyclonal antibody for 90 min. After being washed, the plates were incubated with peroxidase-conjugated goat anti-rabbit IgG for 90 min. After repeated washings, spots representing IFN-γ-producing cells were developed by the addition of 100 μl of 3-amino-9-ethylcarvazole (0.27 mg/ml) in 0.1 M phosphate-citrate buffer (pH 5.0). The spots were counted under a dissecting microscope.

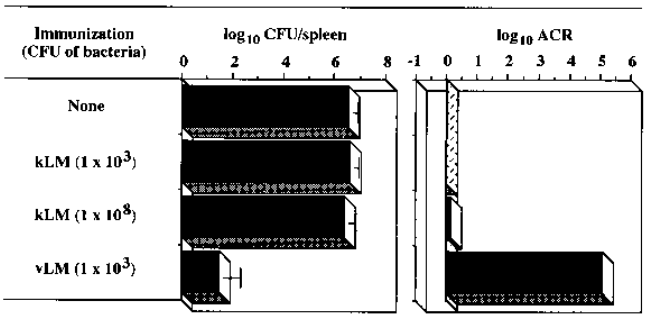

FIG. 1. Protective immunity induced by immunization with viable *L. monocytogenes* (vLM) or killed *L. monocytogenes* (kLM). Six days after immunization with vLM (10^3 CFU) or kLM (10^3 or 10^8 CFU), mice were challenged with 10^4 CFU of vLM and the number of CFU in spleens was determined 2 days later. Acquired cellular resistance (ACR) is expressed as the log difference from nonimmune controls.

Adoptive transfer of acquired cellular resistance (ACR). Non-nylon-wool-adherent T cells (10^7) were intravenously injected into normal sex-matched syngeneic mice, and the recipient mice were challenged with 2×10^4 CFU of viable *L. monocytogenes*. The numbers of viable bacteria in spleens were determined 2 days later by plating dilutions of spleen homogenates onto tryptic soy agar.

Flow cytometric analysis. To determine the population of $CD4^+$ and $CD8^+$ T cells, spleen cells were stained with both phycoerythrin-conjugated anti-CD4 MAb and fluorescein isothiocyanate-conjugated anti-CD8 MAb (Becton Dickinson, Mountain View, Calif.). For analysis of the population of double-negative T cells, cells were stained with fluorescein isothiocyanate-conjugated anti-CD3 MAb, phycoerythrin-conjugated anti-CD4 MAb, and phycoerythrin-conjugated anti-CD8 MAb (Becton Dickinson). Cells were subjected to two-color flow cytometric analysis by using a FACScan (Becton Dickinson).

Statistics. The statistical significance of the data was determined by Student's *t* test, and $P < 0.05$ was taken as significant.

RESULTS

Acquired resistance is induced only by immunization with viable *L. monocytogenes*. Mice were intravenously immunized with viable or killed *L. monocytogenes*. Six days after the immunization, mice were challenged with 10^4 CFU of viable bacteria, and the CFU in spleens were enumerated 2 days later. As shown in Fig. 1, mice acquired protective immunity when immunized with viable bacteria, while protective immunity was not generated when mice were immunized with killed bacteria. Killed bacteria failed to induce protective immunity even at a 5 $\log_{10}$-fold higher dose than viable bacteria.

Difference in the induction of IFN-γ gene expression and production between viable and killed *L. monocytogenes*. One day after injection of mice with killed or viable *L. monocytogenes*, mice were sacrificed and the total RNA was extracted from spleens. RT-PCR was carried out to detect the expression of IFN-γ mRNA. A significant level of PCR products specific for IFN-γ was detected when mice were infected with viable bacteria, whereas the products were hardly observed after injection even with a very high dose of killed bacteria (Fig. 2). The IFN-γ mRNA could not be detected in spleens at 6 and 12 h after injection of mice with killed *L. monocytogenes* (data not shown). Two days after infection with viable bacteria,

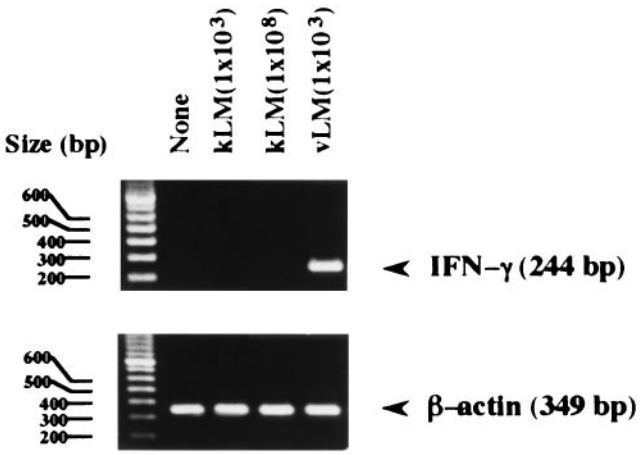

FIG. 2. Expression of IFN-γ mRNA after immunization with viable or killed *L. monocytogenes*. Mice were immunized with 10^3 CFU of viable *L. monocytogenes* or either 10^3 or 10^8 CFU of killed *L. monocytogenes* for 24 h. Total cellular RNA in spleens was extracted and RT-PCR was done by using a specific primer set for β-actin and IFN-γ. Bands in the left lane indicate the positions of the DNA size markers.

IFN-γ could be detected in the sera, whereas IFN-γ production was not observed after injection with killed bacteria (Fig. 3). Based on these observations, we postulated that the initial production of IFN-γ plays a role in the induction of protective immunity against *L. monocytogenes*.

Effect of neutralization of IFN-γ on the generation of IFN-γ-producing protective T cells. In order to determine whether the initial production of IFN-γ plays a role in the induction of protective immunity, mice were immunized with viable *L. monocytogenes* and the IFN-γ production at the initial stage was neutralized by injection with anti-IFN-γ MAb at −2, 24, and 48 h. The IFN-γ titer in sera on day 2 decreased to an undetectable level in mice which were given the MAb (data not shown). Six days after the immunization, mice were sacrificed and splenic T cells were restimulated with killed *L. monocytogenes* (specific antigens) in the presence of normal splenic adherent cells. IFN-γ titer in the supernatant was determined after stimulation for 24 h. A high IFN-γ titer was observed when immune mice were given normal rat IgG, whereas IFN-γ production decreased to the nonimmune control level when mice were treated with anti-IFN-γ MAb at the initial stage of immunization (Fig. 4). To confirm whether the decreased level of IFN-γ production is due to the impaired generation of IFN-γ-producing T cells, the number of *L. monocytogenes*-specific IFN-γ-producing cells was determined by ELISPOT assay. A large number of IFN-γ-producing T cells were generated when mice were immunized with *L. monocytogenes* and treated with normal rat IgG. In contrast, the number of IFN-γ-producing T cells from mice treated with anti-IFN-γ MAb was almost the same as that from normal mice (Fig. 4). We and others reported that protective T cells against *L. monocytogenes* (9, 34, 38) and *M. bovis* BCG (13) are characterized by the IFN-γ-producing ability of T cells. The present results have shown that the induction of protective T cells was significantly impaired by neutralization of the initial production of IFN-γ.

Adoptive transfer of ACR. To finally confirm the function of initially produced IFN-γ, T cells were adoptively transferred into naive mice. T cells from mice immunized with *L. monocytogenes* and treated with anti-IFN-γ MAb or normal rat IgG were transferred into naive mice by intravenous injection, and the recipient mice were challenged with a sublethal dose of *L. monocytogenes*. The resistance to challenge was measured by enumeration of bacterial numbers in the spleens and livers of the recipient mice on day 2. As shown in Fig. 5, growth of bacteria was inhibited in the group that received T cells from immune mice treated with normal rat IgG. In contrast, the bacteria grew rapidly in the spleens and livers of mice given T cells from anti-IFN-γ MAb-treated immune mice. Interestingly, bacteria grew more rapidly in the anti-IFN-γ MAb-treated group than in the nonimmune group. These results showed that R4-6A2 MAb, a neutralizing anti-IFN-γ antibody, completely blocked the generation of protective immunity against *L. monocytogenes.*

It should be ruled out that the observed results were simply due to a high burden of bacteria which may damage the whole spleen in the group treated with anti-IFN-γ MAb. We counted the numbers of bacteria in spleens on day 2 and day 3 after the immunization. No significant difference was observed in the growth of bacteria used for immunization between groups of mice treated with anti-IFN-γ MAb or normal rat IgG. On day 2 after immunization there were 5.6 ± 0.2 and 5.5 ± 0.1 $\log_{10}$ CFU per spleen in mice treated with normal rat IgG and anti-IFN-γ MAb, respectively. The bacterial numbers on day 3 were 6.0 ± 0.2 and 6.1 ± 0.1 $\log_{10}$ CFU per spleen for the respective two groups of mice.

Finally, we have examined the effect of treatment with anti-IFN-γ on the status of spleens in terms of T cell subset by using flow cytometry. Neutralization of initial IFN-γ affected the $CD4^+$ T-cell population rather than the $CD8^+$ T-cell population (Table 1). It appeared that the $CD4^+$ T-cell population is dependent on the IFN-γ endogenously produced at the initial stage of immunization.

DISCUSSION

The present results have clearly shown that protective T cells cannot be generated in the absence of endogenous IFN-γ at the initial stage of immunization with viable *L. monocytogenes*. The administration of anti-IFN-γ neutralizing antibody three times at −2, 24, and 48 h after immunization resulted in almost

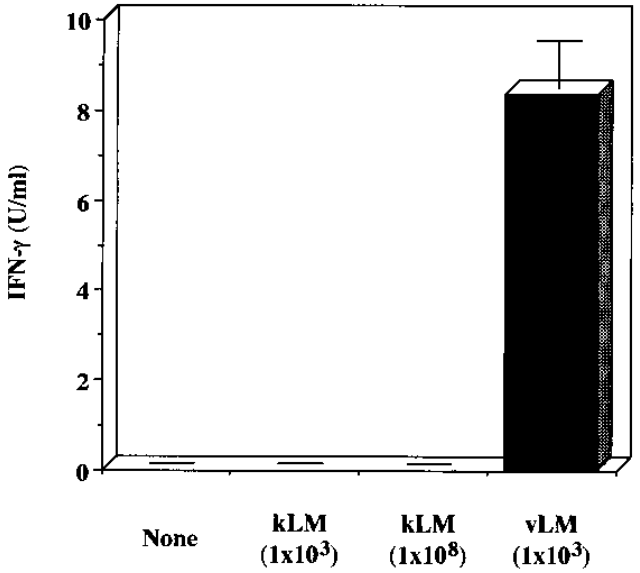

FIG. 3. IFN-γ titers in sera after immunization with viable *L. monocytogenes* (vLM) or killed *L. monocytogenes* (kLM). Mice were immunized with vLM (10^3 CFU) or kLM (10^3 or 10^8 CFU) for 2 days. The titers of IFN-γ in the sera were determined by ELISA.

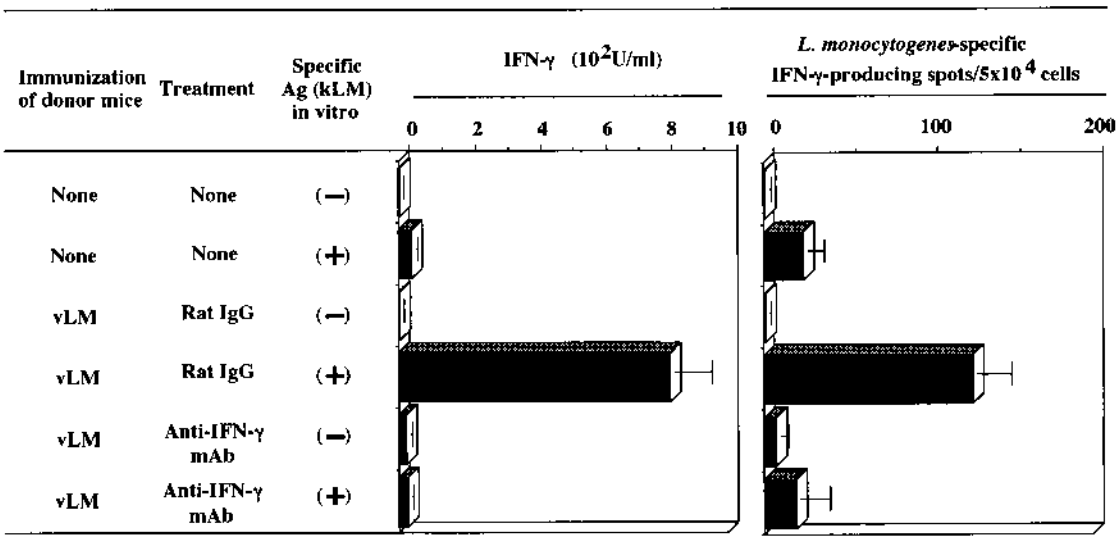

FIG. 4. The effect of IFN-γ neutralization on the generation of antigen-specific, IFN-γ-producing T cells. Mice were immunized with viable *L. monocytogenes* (vLM) and treated with anti-IFN-γ MAb or control rat IgG. Six days later, non-nylon-wool-adherent T cells in spleens were cultured with or without killed *L. monocytogenes* (kLM) (10^8 CFU/ml) in the presence of APC for 24 h, and the supernatants were harvested. IFN-γ titers in the supernatants were determined by ELISA. The antigen-specific, IFN-γ-producing T cells were enumerated by ELISPOT assay. Specific Ag, specific antigen.

a complete abolishment of the in vivo generation of protective T cells, as determined by the adoptive transfer of T cells and the ELISPOT assay for the number of *L. monocytogenes*-specific IFN-γ-producing T cells.

As we have reported previously, viable-bacterium-induced protective $CD4^+$ T cells are characterized by the ability to produce a large amount of IFN-γ upon restimulation with specific antigen (13, 38). Most naive $CD4^+$ T cells may differentiate into either IFN-γ-producing Th1 or interleukin-4 (IL-4)-producing Th2 subsets. One typical example of Th1-dependent protective immunity is the model of *Leishmania major* infection in mice. Adoptive transfer of *L. major*-specific Th1 lines protected mice from infection, while *L. major*-specific Th2 lines not only failed to protect mice from infection but also exacerbated the disease (10, 33). It is generally accepted that the generation of IFN-γ-producing $CD4^+$ T cells is indispensable for the acquisition of specific immunity to various kinds of intracellular parasites.

With respect to the Th1 type of T-cell differentiation, the important role of IFN-γ has been elucidated in a variety of experimental systems. Gajewski et al. noted that IFN-γ inhibited the proliferation of Th2 but not Th1 clones (5), and the presence of IFN-γ in the culture medium resulted in the induction of Th1 clones (6). Scott reported that anti-IFN-γ MAb treatment abrogated the Th1-dependent resistance to *L. major* in C3H/HeN mice and resulted in an enhanced production of IL-4 and IL-5 and a decreased production of IFN-γ by cells taken from these mice. Conversely, cells from BALB/c mice inoculated with parasites plus IFN-γ produced significantly higher levels of IFN-γ and decreased levels of IL-4 and IL-5 (32). Macatonia et al. have also suggested that IFN-γ is im-

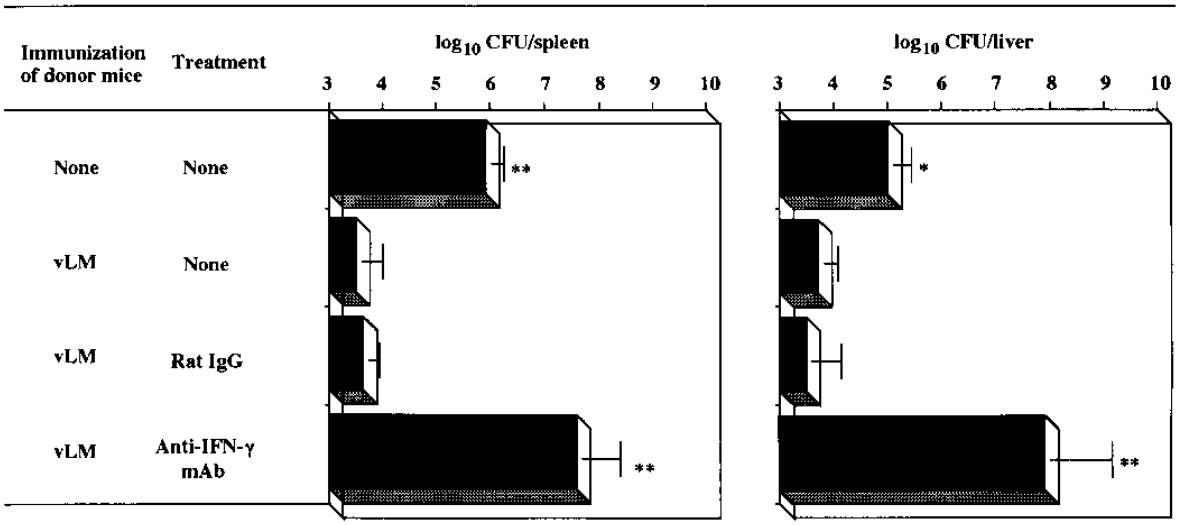

FIG. 5. Expression of acquired cellular resistance by adoptive transfer of T cells. T cells from immune mice (as described in the legend for Fig. 4) were transferred into naive mice, and the mice were challenged with 2×10^4 CFU of viable *L. monocytogenes* (vLM). The number of bacteria in spleens and livers was counted 2 days later. Data are representative of three similar experiments. **, $P < 0.001$ compared with no treatment or rat IgG control; *, $P < 0.01$ compared with no treatment or rat IgG control.

TABLE 1. Flow cytometric analysis of T cells in the spleen[a]

Treatment after immunization	T-cell subset (% of T-cell population in spleen cells)		
	$CD4^+$	$CD8^+$	Double negative
Rat IgG	37.5 ± 3.2	19.2 ± 2.9	6.3 ± 0.8
Anti-IFN-γ MAb	22.5 ± 2.7	12.3 ± 1.6	6.3 ± 1.1

[a] Mice were immunized with viable *L. monocytogenes* cells and treated with anti-IFN-γ MAb or normal rat IgG at −2, 24, and 48 h. The T-cell population in spleen cells was determined on the day of transfer (day 6).

portant in priming the T cells to differentiate into IFN-γ-producing Th1 cells, because anti-IFN-γ antibody diminished the appearance of IFN-γ-producing T cells in priming culture in vitro (17). These results may have implied that the same mechanism is also involved in the differentiation of the *L. monocytogenes*-specific Th1 type of protective T cells; however, there has been no concrete evidence. The present result may be the first confirmation of IFN-γ-dependent generation of protective T cells in *L. monocytogenes* infection.

It may be plausible that the neutralization of early IFN-γ not only affected the generation of protective Th1 cells but also enhanced the Th2 development, because anti-IFN-γ MAb treatment resulted in the exacerbation of challenge infection over the control level in our result. This is consistent with a recent report showing the possibility that IFN-γ depletion results in the polarization of T-cell response into the IL-4-producing Th2 phenotype (25).

Though it is a general fact that killed bacteria are incapable of inducing T-cell-mediated protective immunity, the precise mechanism has been unclear. The present result also suggested that the absence of endogenous IFN-γ production may explain why immunization with killed bacteria is ineffective in the in vivo generation of protective immunity. In mice immunized with killed *L. monocytogenes*, neither the expression of mRNA nor a significant level of production of IFN-γ was observed. In contrast with this result, seemingly controversial data has been reported. Bancroft et al. showed that a heat-killed *Listeria* sp. induced IFN-γ production from spleen cells in vitro (1). Here we do not deny that killed a *Listeria* sp. may be able to stimulate normal spleen cells nonspecifically to produce some amount of IFN-γ in vitro. Besides, we also showed that a low level of IFN-γ was induced after normal spleen cells were stimulated with killed *L. monocytogenes* (Fig. 4). The point of interest in the present study is the significant difference in the IFN-γ-inducing ability between viable and killed bacteria. In any case, whether the inability of killed bacteria to induce protective T cells depends only upon the absence of IFN-γ should be confirmed by a further study using exogenous IFN-γ and killed bacteria.

The development of IFN-γ-producing T cells and protective immunity against intracellular pathogens requires, in some cases, the coordinate action of IFN-γ and IL-12. It was reported that IL-12 primed the differentiation of naive $CD4^+$ T cells into IFN-γ-producing Th1 cells, while IFN-γ production was blocked by the additional administration of anti-IFN-γ MAb (17, 31). In the case of listeriosis, neutralization of IL-12 decreased the resistance to *L. monocytogenes* in SCID mice; however, this decreased resistance was reversed by the administration of recombinant IFN-γ (37). In this regard, the recent report by Miller et al. (20) appears critically relevant to the present finding. They have shown that protective immunity against *L. monocytogenes* could be generated with nonviable antigen when administered with recombinant IL-12. IL-12 is known to be capable of inducing IFN-γ (3); therefore, it is plausible that IL-12 induced IFN-γ and that the IFN-γ alone or in combination with IL-12 then served as the final cytokine(s) to promote the functional differentiation of antigen-primed T cells into Th1 effector cells.

The production of endogenous IFN-γ at the early stage of primary infection with *L. monocytogenes* has been known. Nakane et al. have shown that a significant level of IFN-γ production occurred within 24 to 48 h of infection (24, 26). The serum IFN-γ level observed in our study is consistent with their findings. By using neutralizing antibody to IFN-γ, they demonstrated that the initial, endogenously produced IFN-γ is effective in natural resistance by limiting bacterial growth. To avoid the effect of the anti-IFN-γ MAb on the growth of bacteria used for immunization, we have reduced the immunizing dose. In the present experiment, there was no significant difference in bacterial growth between groups with or without IFN-γ neutralization. In spite of the possible contribution of endogenous IFN-γ at the initial stage to natural resistance, mice do not begin to clear infection until day 4 or 5, when the T-cell response reaches the peak. The present study has demonstrated that the role of endogenous IFN-γ produced at the initial phase is not only for natural resistance but also for the acquisition of antigen-specific protective immunity.

There are several candidates for the cells responsible for the initial production of IFN-γ. Both NK cells and γδ T cells are reported to be capable of producing IFN-γ at the early stage after infection with intracellular pathogens (4, 30). We have found that anti-asialo GM1 treatment of spleen cells abolished the in vitro production of IFN-γ after stimulation with viable *L. monocytogenes* (27), indicating the possibility that NK cells are the main source of early endogenous IFN-γ. Our recent report on NK cell-dependent IFN-γ production by *M. bovis* BCG-stimulated spleen cells also supports this possibility (40).

In the present study, we have applied a short-term administration of anti-IFN-γ antibody intending to neutralize the endogenous IFN-γ only at the initial stage of immunization. A similar but technically different approach has been reported by Harty and Bevan using IFN-γ gene knockout ($GKO^{-/-}$) mice (7). Our result is consistent with their finding that $CD4^+$ protective immunity was not generated in $GKO^{-/-}$ mice. A critical difference is that $CD8^+$ protective T-cell lines could be generated in $GKO^{-/-}$ mice, when mice were immunized with a high dose of an attenuated strain of *L. monocytogenes*. Though the reason why such protective cytolytic T cells could not be generated in our system remains unclear, the application of attenuated or avirulent bacterial strains in immunization is an interesting approach which we should take into consideration. The contribution of cytolytic $CD8^+$ T cells to protection of mice against *L. monocytogenes* is still open for discussion. Harty et al. reported that a listeriolysin O-specific $CD8^+$ T-cell clone established in vitro conferred protection in recipient mice in an IFN-γ-independent fashion (8). Another paper by Lukacs and Kurlander showed a superior role of Lyt-2^+ ($CD8^+$) cells to L3T4^+ ($CD4^+$) cells by negative selection (16). Though these lines of evidence may suggest a pivotal role of $CD8^+$ T cells, a significant contribution of $CD4^+$ T cells to the acquired protective immunity has been shown in mice immunized with *L. monocytogenes* (18, 38). Harty et al. also reported that $CD4^+$ T cells induced protective immunity against *L. monocytogenes*, and this protection was IFN-γ dependent (8). Kaufmann et al. showed that L3T4^+ T-cell clones could specifically lyse *L. monocytogenes*-infected macrophages which were stimulated with IFN-γ, and these T-cell clones induced protection in vivo (12). At the present moment, it appears that both $CD4^+$ and $CD8^+$ T cells could be involved in the protection against *L. monocytogenes*.

In any case, our findings demonstrate that the endogenous IFN-γ at the initial stage of immunization, which is induced only by viable *L. monocytogenes*, not only acts as a major mediator in the expression of primary host defense and protective immunity but also plays a critical role in the induction of protective T cells and protective immunity against *L. monocytogenes*. This study also suggested that IFN-γ, alone or in combination with IL-12, might be a useful adjuvant for vaccination with nonviable bacteria against intracellular pathogens.

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